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- (71) Applicant and  
(72) Inventor: **LINDNER, Marie** [US/US]; 631 Hollow Road,  
Radnor, PA 19087 (US).
- (72) Inventors; and  
(75) Inventors/Applicants (for US only): **MERISKO-LIV-ERSIDGE, Elaine** [US/US]; 258 Colwyn Terr., West Chester, PA 19380 (US). **GOTTARDY, Greta** [US/US]; 204 Marlbrook Lane, Lansdale, PA 19446 (US).
- (74) Agents: **SIMKIN, Michele, M.** et al.; Foley & Lardner, 3000 K Street, N.W., Suite 500, Washington, DC 20007-5143 (US).
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(54) Title: METHOD FOR HIGH THROUGH PUT SCREENING USING A SMALL SCALE MILL OR MICROFLUIDICS

(57) Abstract: The present invention is directed to a high throughput screening (HTS) method, comprising reducing the particle size of a poorly soluble candidate compound to about 1 micron or less using a small scale mill or microfluidics. The product produced from this process is a dispersion of a nanoparticulate candidate compound having one or more surface stabilizers adsorbed onto the surface of the compound. The reduction in particle size results in an increase in the solubility and/or dispersibility of the compound, thus increasing the effectiveness of HTS conducted in conjunction with the particle size reduction process. The particle size reduction process can be conducted before HTS to make screening compounds soluble and/or dispersible, or after HTS to validate an insoluble or poorly compound determined to be active after screening.

## METHOD FOR HIGH THROUGH PUT SCREENING USING A SMALL SCALE MILL OR MICROFLUIDICS

The invention is directed to a method of high throughput screening comprising  
5 reducing the particle size of a poorly soluble compound using a small scale mill or  
microfluidics to increase the solubility and/or dispersibility of the compound.

### BACKGROUND

#### 10 A. Background Relating to High Throughput Screening

Drug discovery relies on the ability to identify compounds that interact with a  
selected target, such as cells, an antibody, receptor, enzyme, transcription factor, or the  
like. Traditional drug discovery relied on collections or "libraries" obtained from  
proprietary databases of compounds accumulated over many years, natural products,  
15 fermentation broths, and rational drug design. Recent advances in molecular biology,  
chemistry, and automation have resulted in the development of rapid, HTS protocols to  
screen these collections. HTS and sample preparation can account for about 1% (about  
US\$2.7 million) of developing a drug. D. McName, "Robotised assays," *Lancet*, 346:  
114 (1995).

20 The beneficial effects of combinatorial chemistry and HTS are just beginning to  
be felt at the later stages of the drug pipeline. Some 40 drugs have emerged from HTS  
and made it to clinical trials. Directors from 50 HTS laboratories, participating in the  
study "High-Throughput Screening 2000: New Trends and Directions," identified 46  
drug candidates that originated in their HTS laboratories, and which are being tested in  
25 humans. The backlog of new chemical entities to be screened is monumental, and the  
robots will continue to assay compounds, 24/7. "Screening," *Drug Discovery/  
Technology News*, 4 (2001).

Lab directors are seeking technologies to facilitate higher throughput, reduce the  
use of scarce compounds, cells, membranes, and reagents, and to lower reagent costs.  
30 New technologies in HTS have significantly increased throughput and reduced assay

volumes. Key advances over the past few years include new fluorescence methods, detection platforms, and liquid-handling technologies. Screening 100,000 samples per day in miniaturized assay volumes will soon become routine. Hertzberg et al., "High-throughput screening: new technology for the 21st century," *Curr. Opin. Chem. Biol.*, 5 4:445-51 (2000).

#### B. Solubility of Drug Candidates

The solubility behavior of drugs remains one of the most challenging aspects in formulation development. Leuner et al., "Improving drug solubility for oral delivery using solid dispersions," *Eur. J. Pharm. Biopharm.*, 50:47-60 (2000). With the advent of combinatorial chemistry and HTS, the number of poorly soluble compounds has dramatically increased. Although solid solutions have tremendous potential for improving drug solubility, forty years of research have resulted in only a few marketed 15 products using this approach. *Id.*

The determination of solubility or dispersibility in a HTS environment is invaluable in the selection of the most promising potential drug candidates. This is because the level of permeability or solubility needed for oral absorption is related to potency. The relative importance of poor solubility and poor permeability towards the 20 problem of poor oral absorption depends on the research approach used for lead generation. Current research approaches tend to result in a large number of poorly soluble drug candidates. For example, a "rational drug design" approach leads to time-dependent higher molecular weight, higher H-bonding properties, unchanged lipophilicity, and, hence, poorer permeability. Similarly, a HTS-based approach leads to 25 higher molecular weight, unchanged H-bonding properties, higher lipophilicity, and, hence, poorer aqueous solubility. *Id.*

One method used to determine the solubility of potential drug candidates (usually from combinatorial chemistry) prior to HTS is based on laser nephelometry that can be supplied as dimethyl sulfoxide (DMSO) solutions in 96-well plates. Bevan et al., 30 "A high-throughput screening method for the determination of aqueous drug solubility

using laser nephelometry in microtiter plates," *Anal. Chem.*, 72:1781-7 (Apr. 15, 2000). However, this method does not increase the solubility of a drug candidate, as it merely determines whether the drug is sufficiently soluble for further study.

Another method of increasing the solubility of a compound prior to HTS is to  
5 dissolve the compound in a solvent, although such a solvent can be toxic and can interfere with the activity of the compound.

Yet another method which can be used to increase solubility of a compound, but which to the best of Applicants' knowledge has not been used in conjunction with HTS, is microfluidics. While microfluidics may be employed to obtain small particles, it is  
10 not practical for larger amounts of compounds and has many inherent complications. For preparation of many screening trays for HTS, or for preparation of larger amounts of compound for use in validation of active screens, microfluidics is not appropriate. In addition, since microfluidics does not allow for stabilization of small particles, particles reduced to a nanoparticulate particle size with microfluidics must be used immediately  
15 to prevent particle size growth via agglomeration and recrystallization. In addition, compounds prepared using microfluidics cannot be scaled up for later research.

### C. Milling of Pharmaceutical Compositions

20 Pharmaceutical agents that exhibit poor solubility often can diminish the efficacy of a drug formulation. Improved solubility can be achieved by reducing a drug's particle size, which increases its surface area. The micronization method of grinding drug compounds to achieve a smaller particle size is well established. To the best of Applicants' knowledge, milling of pharmaceutical products has not been used in  
25 conjunction with HTS.

Conventional milling techniques, such as jet mill or rotor stator colloid mills, grind drugs into powders that have particle sizes ranging from 0.1  $\mu\text{m}$  to 25  $\mu\text{m}$ . Wet media mills, such as the ones described in U.S. Patent Nos. 5,797,550 issued to Woodall et al. and 4,848,676 issued to Stehr, are generally used to mill or grind relatively large  
30 quantities of materials. These rather large media mills are not generally suitable for

grinding small or minute quantities, such as that required for samples to be used in or generated from HTS. U.S. Patent No. 5,593,097 issued to Corbin recognizes the need for milling small quantities, as small as 0.25 grams, to a size less than 0.5 micron to about 0.05 micron (average diameter) in about 60 minutes.

5        There are several research groups and companies developing and manufacturing micro-, mini-, and nanomills. For example, W.A. Bachofen, in Switzerland manufactures the DYNO<sup>®</sup>-Mill, a continuously operating bead mill with a horizontal grinder container. Bachofen make a variety of DYNO<sup>®</sup>-Mills with different specifications, such as a small laboratory model (DYNO<sup>®</sup>-Mill KDL A) which  
10 accommodates 0.15 – 0.3 liter grinding containers for discontinuous operation, and 0.3 – 0.6 liters in continuous operation. The grinding beads are spherical and have a diameter of 0.2 – 1.5 mm. The power output of the mill motor is 1.5 – 1.85 kW. One of the preferred application fields for this particular DYNO<sup>®</sup>-Mill is for mechanical cell disruption in microbiology and biochemistry. At the other end of the size and volume  
15 range is the DYNO<sup>®</sup>-Mill KD 600 that has grinders with a volume capacity of 600 liters.

A specially developed, high efficiency, bead mill for dispersion and wet grinding applications uses Bachofen's "newly developed DYNO<sup>®</sup> accelerators" (DYNO<sup>®</sup>-Mill ECM). The ECM-Pilot version accommodates 1.5 liters and has a motor output of 6.8 –  
20 7.5 kW; the ECM-Pro model has a capacity of 18.2 liters and outputs 36 – 45 kW. In addition, the company also has an apparatus (TURBULA<sup>®</sup>) that mixes powdery substances with differing specific weights and particle sizes, and is convenient for use in the pharmaceutical industry.

Netzsch, Inc. make the LMZ Zeta System, which has a high energy, high flow,  
25 multiple pass grinding mechanism to achieve very narrow submicron size particles. Their Dynamic Cartridge Media Separator<sup>™</sup> (DCMS) allows the use of grinding media as small as 100  $\mu$ m in size. The different models can accommodate from 1.6 liters to 62 liters of suspension. One model, the MiniZeta is a high energy grinding system for small batch analysis. In this particular model, the batch size is down to 250 ml with a

chamber volume of 300 ml. Yet another, the Laboratory Attrition Mill is designed for very small quantities of material, wherein the grinding vessel is jacketed for cooling or heating.

MicroGrinding Systems, Inc. have made a Vibrokinetic Energy Grinding Mill, 5 which is an "extremely fast and very energy efficient" milling machine that can be operated either wet or dry. This particular mill uses a unique tuned spring system to suspend the grinding chamber and motor energy source. This saves and reuses "rebound" energy and makes the mill cost-effective and maintenance-free, especially since the motor is the only moving part, so energy expenditure and power maintenance 10 are minimal. Adjustable air cyclone classifiers separate product streams in the 5-10 micron range.

The mill is available in several basic models, including a Laboratory Mill "capable of producing 50 pounds per hour of fine product from a ¼" feed, and a Pilot Plant Mill which produces 250 pounds per hour of fine powder from a ¼" hard feed 15 material. The company suggests pharmaceuticals can be ground using these apparatus.

Nanoscale Combinatorial Synthesis, Inc. (Nanosyn) is publicizing their Accelerated Nanoscale Synthesis Technology (ANST<sup>™</sup>) technology, which enables screening of compounds in miniaturized assays. Their proprietary products and services were publicized in January, 2001 when the company announced it will provide small 20 molecule libraries to Euroscreen, a Belgium-based molecular diagnostic company.

Finally, a small scale mill exhibiting improvements over prior art technology is described in U.S. Provisional Application Serial No. 60/137,142, filed on June 1, 1999, and U.S. utility Application No. 09/583,893, filed on May 31, 2000, which are specifically incorporated by reference.

25 Milling of pharmaceutical or diagnostic agents to a submicron particle size is described, for example, in U.S. Patent Nos. 5,145,684 "for Surface Modified Drug Nanoparticles;" 5,298,262 for "Use of Ionic Cloud Point Modifiers to Prevent Particle Aggregation During Sterilization;" 5,302,401 for "Method to Reduce Particle Size Growth During Lyophilization;" 5,318,767 for "X-Ray Contrast Compositions Useful in Medical

Imaging;" 5,326,552 for "Novel Formulation For Nanoparticulate X-Ray Blood Pool Contrast Agents Using High Molecular Weight Non-ionic Surfactants;" 5,328,404 for "Method of X-Ray Imaging Using Iodinated Aromatic Propanedioates;" 5,336,507 for "Use of Charged Phospholipids to Reduce Nanoparticle Aggregation;" 5,340,564 for  
5 "Formulations Comprising Olin 10-G to Prevent Particle Aggregation and Increase Stability;" 5,346,702 for "Use of Non-Ionic Cloud Point Modifiers to Minimize Nanoparticulate Aggregation During Sterilization;" 5,349,957 for "Preparation and Magnetic Properties of Very Small Magnetic-Dextran Particles;" 5,352,459 for "Use of Purified Surface Modifiers to Prevent Particle Aggregation During Sterilization;"  
10 5,399,363 for "Surface Modified Anticancer Nanoparticles;" 5,401,492 for "Water Insoluble Non-Magnetic Manganese Particles as Magnetic Resonance Enhancement Agents;" 5,429,824 for "Use of Tyloxapol as a Nanoparticulate Stabilizer;" 5,447,710 for "Method for Making Nanoparticulate X-Ray Blood Pool Contrast Agents Using High Molecular Weight Non-ionic Surfactants;" 5,451,393 for "X-Ray Contrast  
15 Compositions Useful in Medical Imaging;" 5,466,440 for "Formulations of Oral Gastrointestinal Diagnostic X-Ray Contrast Agents in Combination with Pharmaceutically Acceptable Clays;" 5,470,583 for "Method of Preparing Nanoparticle Compositions Containing Charged Phospholipids to Reduce Aggregation;" 5,472,683 for "Nanoparticulate Diagnostic Mixed Carbamic Anhydrides as X-Ray Contrast Agents  
20 for Blood Pool and Lymphatic System Imaging;" 5,494,683 for "Surface Modified Anticancer Nanoparticles;" 5,500,204 for "Nanoparticulate Diagnostic Dimers as X-Ray Contrast Agents for Blood Pool and Lymphatic System Imaging;" 5,518,187 for "Method of Grinding Pharmaceutical Substances;" 5,518,738 for "Nanoparticulate NSAID Formulations;" 5,521,218 for "Nanoparticulate Iododipamide Derivatives for  
25 Use as X-Ray Contrast Agents;" 5,525,328 for "Nanoparticulate Diagnostic Diatrizoxy Ester X-Ray Contrast Agents for Blood Pool and Lymphatic System Imaging;" 5,543,133 for "Process of Preparing X-Ray Contrast Compositions Containing Nanoparticles;" 5,552,160 for "Surface Modified NSAID Nanoparticles;" 5,560,931 for "Formulations of Compounds as Nanoparticulate Dispersions in Digestible Oils or Fatty

Acids;" 5,565,188 for "Polyalkylene Block Copolymers as Surface Modifiers for Nanoparticles;" 5,569,448 for "Sulfated Non-ionic Block Copolymer Surfactant as Stabilizer Coatings for Nanoparticle Compositions;" 5,571,536 for "Formulations of Compounds as Nanoparticulate Dispersions in Digestible Oils or Fatty Acids;" 5 5,573,749 for "Nanoparticulate Diagnostic Mixed Carboxylic Anhydrides as X-Ray Contrast Agents for Blood Pool and Lymphatic System Imaging;" 5,573,750 for "Diagnostic Imaging X-Ray Contrast Agents;" 5,573,783 for "Redispersible Nanoparticulate Film Matrices With Protective Overcoats;" 5,580,579 for "Site-specific Adhesion Within the GI Tract Using Nanoparticles Stabilized by High Molecular Weight, Linear Poly(ethylene Oxide) Polymers;" 5,585,108 for "Formulations of Oral Gastrointestinal Therapeutic Agents in Combination with Pharmaceutically Acceptable Clays;" 5,587,143 for "Butylene Oxide-Ethylene Oxide Block Copolymers Surfactants as Stabilizer Coatings for Nanoparticulate Compositions;" 5,591,456 for "Milled Naproxen with Hydropropyl Cellulose as Dispersion Stabilizer;" 5,593,657 for "Novel 15 Barium Salt Formulations Stabilized by Non-ionic and Anionic Stabilizers;" 5,622,938 for "Sugar Based Surfactant for Nanocrystals;" 5,628,981 for "Improved Formulations of Oral Gastrointestinal Diagnostic X-Ray Contrast Agents and Oral Gastrointestinal Therapeutic Agents;" 5,643,552 for "Nanoparticulate Diagnostic Mixed Carbonic Anhydrides as X-Ray Contrast Agents for Blood Pool and Lymphatic System Imaging;" 20 5,718,388 for "Continuous Method of Grinding Pharmaceutical Substances;" 5,718,919 for "Nanoparticles Containing the R(-)Enantiomer of Ibuprofen;" 5,747,001 for "Aerosols Containing Beclomethasone Nanoparticle Dispersions;" 5,834,025 for "Reduction of Intravenously Administered Nanoparticulate Formulation Induced Adverse Physiological Reactions;" 6,045,829 "Nanocrystalline Formulations of Human 25 Immunodeficiency Virus (HIV) Protease Inhibitors Using Cellulosic Surface Stabilizers;" 6,068,858 for "Methods of Making Nanocrystalline Formulations of Human Immunodeficiency Virus (HIV) Protease Inhibitors Using Cellulosic Surface Stabilizers;" 6,153,225 for "Injectable Formulations of Nanoparticulate Naproxen;" 6,165,506 for "New Solid Dose Form of Nanoparticulate Naproxen;" and 6,221,400 for

“Methods of Treating Mammals Using Nanocrystalline Formulations of Human Immunodeficiency Virus (HIV) Protease Inhibitors;” 6,264,922 for “Nebulized Aerosols Containing Nanoparticle Dispersions;” 6,267,989 for “Methods for Preventing Crystal Growth and Particle Aggregation in Nanoparticle Compositions;” 6,270,806 for “Use of 5 PEG-Derivatized Lipids as Surface Stabilizers for Nanoparticulate Compositions;” and 6,316,029 for “Rapidly Disintegrating Solid Oral Dosage Form,” all of which are specifically incorporated by reference. In addition, U.S. Patent Application No. 20020012675 A1, published on January 31, 2002, for “Controlled Release Nanoparticulate Compositions,” describes nanoparticulate compositions, and is 10 specifically incorporated by reference.

With the synergistic and multiplicative interactions of rational drug design, recombinant biotechnology, combinatorial chemistry, and HTS, millions of compounds are being synthesized by chemists. However, development of these candidate compounds has often been impeded, if not terminated, due to biopharmaceutic and/or 15 pharmacokinetic constraints related to poor solubility of candidate compounds. This has resulted in delays in development time and escalation of cost in the drug research programs. Panchagnula et al., “Biopharmaceutics and pharmacokinetics in drug research,” *Int. J. Pharm.*, 201:131-50 (May 25, 2000).

The present invention satisfies the need in the art for rapid methods of screening 20 compounds for acceptable bioavailability, such as pharmaceutically acceptable bioavailability, as well as increasing the solubility and/or dispersibility of candidate compounds.

### SUMMARY

25 The present invention is directed to a method of increasing the effectiveness of HTS, comprising reducing the particle size of a poorly soluble candidate compound to about 1 micron or less using a small scale mill or microfluidics.

The product produced from this process is a dispersion of a nanoparticulate candidate compound having one or more surface stabilizers adsorbed onto the surface of 30 the compound. The reduction in particle size results in an increase in the solubility

and/or dispersibility of the candidate compound, thus increasing the effectiveness of HTS conducted in conjunction with the milling or microfluidics process. The particle size reduction process, accomplished via milling or microfluidics, can be conducted before HTS to make screening compounds soluble and/or more dispersible, or after HTS 5 to validate a poorly soluble compound determined to be active after screening. The liquid dispersion resulting from the milling or microfluidics process can be used directly in HTS.

Thus, one embodiment of the invention is directed to a method of HTS comprising milling in a small scale mill one or more poorly soluble candidate 10 compounds to be screened to about 1 micron or less. The milling process can be performed in the presence of at least one surface stabilizer, or at least one surface stabilizer can be added to the compound dispersion following particle size reduction. Such surface stabilizers adsorb to the surface of the candidate compound, and do not chemically interact or alter the compound's properties. Following particle size 15 reduction, the nanoparticulate compound dispersion is run through a standard HTS screen, such as an enzymatic or whole cell assay, to determine if the candidate compound has the desired activity.

Similarly, in another embodiment of the invention, one or more poorly soluble candidate compounds are subjected to microfluidization to reduce the particle size of the 20 compounds to about 1 micron or less. The microfluidization process can be performed in the presence of at least one surface stabilizer, or at least one surface stabilizer can be added to the compound dispersion following microfluidization. Such surface stabilizers adsorb to the surface of the candidate compound, and do not chemically interact or alter the compound's properties. Following particle size reduction, the nanoparticulate 25 compound dispersion is run through a standard HTS screen, such as an enzymatic or whole cell assay, to determine if the candidate compound has the desired activity.

Yet another embodiment of the invention is directed to a method of HTS comprising running one or more poorly soluble candidate compounds through a standard HTS screen, such as an enzymatic or whole cell assay. This is followed by

reducing the particle size of the compounds identified as having the desired activity in a small scale mill, either individually or in mixtures, to about 1 micron or less to increase the solubility and/or dispersibility of the compounds to an acceptable level, such as a pharmaceutically acceptable level. The milling process can be performed in the  
5 presence of at least one surface stabilizer, or at least one surface stabilizer can be added to the compound dispersion following particle size reduction. Such surface stabilizers adsorb to the surface of the candidate compound, and do not chemically interact or alter the compound's properties.

Finally, the invention also encompasses a method of HTS comprising running  
10 one or more poorly soluble candidate compounds through a standard HTS screen, such as an enzymatic or whole cell assay. This is followed by reducing the particle size of compounds identified as having the desired activity via microfluidization, either individually or in mixtures, to about 1 micron or less to increase the solubility and/or dispersibility of the compounds to an acceptable level, such as a pharmaceutically  
15 acceptable level. The microfluidization can be performed in the presence of at least one surface stabilizer, or at least one surface stabilizer can be added to the compound dispersion following microfluidization. Such surface stabilizers adsorb to the surface of the candidate compounds, and do not chemically interact or alter the candidate compound's properties.

20 Both the foregoing general description and the following detailed description are exemplary and explanatory and are intended to provide further explanation of the invention as claimed. Other objects, advantages, and novel features will be readily apparent to those skilled in the art from the following detailed description of the invention.

25

### **DETAILED DESCRIPTION OF THE INVENTION**

Prior to the present invention, particle size has not been taken into account when  
30 preparing compounds for HTS screening or further drug discovery activities. Rather,

compounds with poor solubility and/or dispersibility are dissolved in a solvent, which can be toxic or interfere with the activity of the compound.

An HTS method according to the invention comprises reducing the particle size of one or more poorly soluble candidate compounds to be screened, either individually 5 or in mixtures, to about 1 micron or less using a small scale mill or microfluidics. Following particle size reduction, the nanoparticulate compound dispersion is run through a standard HTS screen, such as an enzymatic or whole cell assay, to identify compounds having a desired activity. The assays can be any known HTS assay, and can be manual or automatic.

10 Alternatively, the invention encompasses a method comprising running a poorly soluble candidate compound through a standard HTS screen, such as an enzymatic or whole cell assay. The assays can be any known HTS assay, and can be manual or automatic. This is followed by reducing the particle size of compounds identified as having a desired activity, either individually or in mixtures, to about 1 micron or less 15 using a small scale mill or microfluidics. This reduction in particle size results in increasing the solubility and/or dispersibility of the compound to an acceptable level, such as a pharmaceutically acceptable level.

### **Dispersion Medium**

20 The candidate compound must be insoluble or poorly soluble in at least one liquid medium. A preferred liquid dispersion medium is water. However, the invention can be practiced with other liquid media in which a candidate compound is poorly soluble and dispersible including, for example, aqueous salt solutions, safflower oil, and solvents such as ethanol, t-butanol, hexane, and glycol. The pH of the dispersion media 25 can be adjusted by techniques known in the art.

### **"Poorly Soluble"**

By "poorly soluble" it is meant that the candidate compound has a solubility in a liquid dispersion medium of less than about 10 mg/ml, and preferably of less than about

1 mg/ml. If a candidate compound is not poorly soluble, it can be conjugated to a salt or other substance to render the compound poorly soluble. Thus, all candidate compounds having, for example, therapeutic, cosmetic, diagnostic, or bioengineering uses are presumed suitable for the invention. The term "candidate compound" is not limited to a  
5 substance having pharmaceutical activity, as the invention is intended to encompass any and all poorly soluble compounds or compounds which can be made poorly soluble, and which has a desired activity, for example, compounds useful in pharmaceuticals, cosmetics, diagnostics, bioengineering, and agriculture, such as pesticides, fertilizers, insecticides, and herbicides.

10 For example, if the candidate compound is soluble in the liquid dispersion medium, the compound can be conjugated to other molecules or moieties to render the compound poorly soluble prior to milling. Compounds can be conjugated to, for example, hydrophobic molecules, molecules with amphipathic properties, lipid  
15 molecules, phospholipid molecules, fats, prenyl groups, or palmitoyl groups to render the candidate compound less soluble or poorly soluble prior to milling or microfluidization. Such conjugation can be through direct conjugation to specific sites on the compound, to the N-terminal or C-terminal residue of the compound via intermediate spacer molecules which can be attached to one or more sites on the compound, and/or through internal side chains on the compound.

20 Furthermore, a compound can be rendered less soluble by addition of amino acid residues either during the chemical synthesis or the biological expression of the compound, in particular, amino acid residues or derivatives with hydrophobic properties. Such residues or motifs can be separated from the compound by hydrolysable linkers or linkers which can be cleaved *in vivo*, for example, by specified  
25 enzymes or esterases.

In addition, the candidate compounds can be conjugated to pharmaceutically acceptable salts to render the compounds poorly soluble. Furthermore, the compounds can be rendered poorly soluble by adjusting the pH of the dispersion medium.

### **Exemplary Milling Methods**

One milling method according to the present invention comprises providing a dispersion of one or more poorly soluble candidate compounds to be milled and attrition milling media. Preferred attrition media has a particle size of 500 microns or less.

5 For an exemplary milling machine, the dispersion is inserted into a vessel, such as a cylindrical or other shaped vessel, and an agitator and a coupling that closes the vessel are provided. The coupling has an opening through which a portion of the agitator extends, and the agitator comprises a cylindrical rotor and a shaft extending therefrom, wherein the cylindrical rotor is dimensioned such that an outer periphery is  
10 minimal, for example, no greater than 3 mm away from an inner surface of the wall, although other size ranges can be employed in the invention and the exemplary amount is not intended to be limiting. An agitator is inserted into the vessel and the coupling is sealed or closed, wherein the amount of dispersion inserted into the vessel is such that the dispersion eliminates substantially all of the air in the vessel when the agitator is  
15 fully inserted into the vessel. The agitator is then rotated for a predetermined period. One or more surface stabilizers for the candidate compound(s) are added to the dispersion either before or after milling.

Another method according to the present invention comprises providing a dispersion comprising one or more poorly soluble candidate compounds to be milled  
20 and attrition milling media. Preferred attrition media has a particle size of 500 microns or less. An agitator having a cylindrical rotor and shaft extending therefrom is provided, the agitator is inserted in a horizontally oriented vessel, and the vessel is sealed. The rotor is dimensioned to provide a minimal gap, for example, no greater than 3 mm between an outer surface of the rotor and an inner surface of the vessel, although other  
25 size ranges can be employed in the invention and the exemplary amount is not intended to be limiting. At least one port through the vessel is provided, and the port is maintained at the highest point of the horizontally oriented vessel. The vessel is filled with the compound dispersion until the dispersion drives out substantially all of the air in the vessel. Finally, the agitator is rotated for a predetermined period. One or more

surface stabilizers for the candidate compound(s) are added to the dispersion either before or after milling.

Because virtually all of the air can be displaced in the vertically and horizontally oriented mills, vortexing and contamination problems are minimized or avoided. Thus, the milling process according to the present invention can prevent the dispersion formulation from foaming.

#### **Exemplary Microfluidization Method**

U.S. Patent No. 5,510,118, for "Method for Preparing Therapeutic Compositions Containing Nanoparticles," describes an exemplary method of making sub-micron sized poorly soluble compounds using microfluidization. This patent is specifically incorporated by reference.

#### **Advantages of the HTS Method of the Invention**

One advantage of the HTS methods of the invention, when the dispersion medium is water, is that for whole cell HTS screens, the milled or microfluidized aqueous compound dispersion of the invention is non-toxic, as water is non-toxic to cells. This is in contrast to prior art methods, in which poorly soluble compounds were solubilized in solvents. As a result, cellular activity is more clearly observed for the dispersions of the invention, since there is no solvent-induced cell toxicity.

Another advantage of the methods of the invention is that the milled or microfluidized dispersions can be used directly in HTS by aliquoting the correct concentration into wells to run through standard HTS screens. Additionally, the concentration can vary between different wells of the HTS assay. Milled and microfluidized compound dispersions can also be used in other enzymatic or cellular tests of activity and toxicity. Again, an advantage of the present invention is that no toxic solvent is present in the milled dispersion. In addition, the compound requires very little reformulation work for clinical studies.

Nanoparticulate dispersions prepared according to the invention are stable for

extensive periods of time, *i.e.*, for a year or more. Thus, the HTS method of the invention does not require immediately screening a compound following milling or microfluidization. Moreover, compounds prepared according to the invention can be readily scaled up for manufacturing.

5       The time required to prepare a milled micro- or nanoparticulate suspension from a given amount of starting material is on average about one hour or less. Thus 3-4 samples, or more, can be comfortably milled within a working day with one small scale mill, including preparation time, milling, harvesting, and particle sizing of the milled dispersion. The time limiting factor is preparation and analyzation of the resulting  
10 sample; with pre-prepped samples, about 6-8 compounds or more could be milled per day in each small scale mill.

In general, the time required for the particle size reduction process is selected from the group consisting of about one hour or less, about 45 minutes or less, about 40 minutes or less, about 35 minutes or less, about 30 minutes or less, about 25 minutes or  
15 less, about 20 minutes or less, about 15 minutes or less, about 10 minutes or less, and about 5 minutes or less.

#### **Attrition Media**

The attrition media used in a small scale mill can be a polymeric type, such as  
20 formed of polystyrene or cross-linked polystyrene having a nominal diameter of no greater than 500 microns. Other particle sizes of useful milling media include 200 microns and 50 microns, and a mixtures of sizes ranging between about 50 and about 500 microns.

U.S. Patent Nos. 5,518,187, 5,718,388, and 5,862,999 disclose milling  
25 pharmaceutical products using polymeric milling media. These patents further disclose dispersion formulations for a wet media milling. The disclosures of these patents are specifically incorporated by reference.

### **Surface Stabilizers**

The one or more surface stabilizers are adsorbed on the surface of the candidate compound in an amount sufficient to maintain the candidate compound at an effective average particle size of less than about 1 micron, or other desired particle size.

- 5        The relative amount of the candidate compound and surface stabilizer can vary widely and the optimal amount of the surface stabilizer can depend, for example, upon the particular candidate compound and surface stabilizer selected, the critical micelle concentration of the surface stabilizer if it forms micelles, *etc.*

The at least one surface stabilizer is present in the liquid dispersion medium in  
10 an amount selected from the group consisting of from about 0.01% to about 90%, about 1% to about 90%, and about 5% to about 90%, by weight, based on the total dry weight of the candidate compound and surface stabilizer. The one or more surface stabilizers can be added to the liquid dispersion medium either before or after size reduction of the one or more candidate compounds.

- 15        Useful surface stabilizers, which are known in the art and described in U.S. Patent No. 5,145,684, specifically incorporated by reference, are believed to include those which physically adhere to the surface of the candidate compound but do not chemically bond to or interact with the compound. Furthermore, the individually adsorbed molecules of the surface stabilizer are essentially free of intermolecular cross-  
20 linkages. Two or more surface stabilizers can be employed in the methods of the invention.

Suitable surface stabilizers can preferably be selected from known organic and inorganic pharmaceutical excipients. Such excipients include various polymers, low molecular weight oligomers, natural products, and surfactants. Preferred surface  
25 stabilizers include nonionic and ionic surfactants.

Representative examples of surface stabilizers include gelatin, casein, lecithin (phosphatides), dextran, gum acacia, cholesterol, tragacanth, stearic acid, benzalkonium chloride, calcium stearate, glycerol monostearate, cetostearyl alcohol, cetomacrogol emulsifying wax, sorbitan esters, polyoxyethylene alkyl ethers (*e.g.*, macrogol ethers

such as cetomacrogol 1000), polyoxyethylene castor oil derivatives, polyoxyethylene sorbitan fatty acid esters (*e.g.*, the commercially available Tweens<sup>®</sup> such as *e.g.*, Tween 20<sup>®</sup> and Tween 80<sup>®</sup> (ICI Speciality Chemicals)); polyethylene glycols (*e.g.*, Carbowaxs 3550<sup>®</sup> and 934<sup>®</sup> (Union Carbide)), polyoxyethylene stearates, colloidal silicon dioxide, 5 phosphates, sodium dodecylsulfate, carboxymethylcellulose calcium, carboxymethylcellulose sodium, methylcellulose, hydroxyethylcellulose, hydroxypropylcellulose, hydroxypropylmethyl-cellulose phthalate, noncrystalline cellulose, magnesium aluminium silicate, triethanolamine, polyvinyl alcohol (PVA), polyvinylpyrrolidone (PVP), 4-(1,1,3,3-tetramethylbutyl)-phenol polymer with ethylene 10 oxide and formaldehyde (also known as tyloxapol, superione, and triton), poloxamers (*e.g.*, Pluronic F68<sup>®</sup> and F108<sup>®</sup>, which are block copolymers of ethylene oxide and propylene oxide); poloxamines (*e.g.*, Tetronic 908<sup>®</sup>, also known as Poloxamine 908<sup>®</sup>, which is a tetrafunctional block copolymer derived from sequential addition of propylene oxide and ethylene oxide to ethylenediamine (BASF Wyandotte Corporation, 15 Parsippany, N.J.)); Tetronic 1508<sup>®</sup> (T-1508) (BASF Wyandotte Corporation), dialkylesters of sodium sulfosuccinic acid (*e.g.*, Aerosol OT<sup>®</sup>, which is a dioctyl ester of sodium sulfosuccinic acid (American Cyanamid)); Duponol P<sup>®</sup>, which is a sodium lauryl sulfate (DuPont); Tritons X-200<sup>®</sup>, which is an alkyl aryl polyether sulfonate (Rohm and Haas); Crodestas F-110<sup>®</sup>, which is a mixture of sucrose stearate and sucrose 20 distearate (Croda Inc.); p-isononylphenoxypoly-(glycidol), also known as Olin-IOG<sup>®</sup> or Surfactant 10-G<sup>®</sup> (Olin Chemicals, Stamford, CT); Crodestas SL-40<sup>®</sup> (Croda, Inc.); and SA9OHCO, which is C<sub>18</sub>H<sub>37</sub>CH<sub>2</sub>C(O)N(CH<sub>3</sub>)-CH<sub>2</sub>(CHOH)<sub>4</sub>(CH<sub>2</sub>OH)<sub>2</sub> (Eastman Kodak Co.); decanoyl-N-methylglucamide; n-decyl β-D-glucopyranoside; n-decyl β-D-maltopyranoside; n-dodecyl β-D-glucopyranoside; n-dodecyl β-D-maltoside; heptanoyl- 25 N-methylglucamide; n-heptyl-β-D-glucopyranoside; n-heptyl β-D-thioglucoside; n-hexyl β-D-glucopyranoside; nonanoyl-N-methylglucamide; n-nonyl β-D-glucopyranoside; octanoyl-N-methylglucamide; n-octyl-β-D-glucopyranoside; octyl β-D-thioglucopyranoside; PEG-phospholipid, PEG-cholesterol, PEG-cholesterol derivative, PEG-vitamin A, PEG-vitamin E, lysozyme, and random copolymers of

vinyl acetate and vinyl pyrrolidone (*i.e.*, Plasdone® S630), and the like.

Most of these surface stabilizers are known pharmaceutical excipients and are described in detail in the *Handbook of Pharmaceutical Excipients*, published jointly by the American Pharmaceutical Association and The Pharmaceutical Society of Great Britain (The Pharmaceutical Press, 1995), specifically incorporated by reference. The surface stabilizers are commercially available and/or can be prepared by techniques known in the art.

#### **Candidate Compound/Surface Stabilizer Particle Size**

The compound of the invention is reduced to an effective average particle size of less than about 1 micron. The compound can also be reduced to an effective average particle size of less than about 900 nm, less than about 800 nm, less than about 700 nm, less than about 600 nm, less than about 500 nm, less than about 400 nm, less than about 300 nm, less than about 250 nm, less than about 200 nm, less than about 150 nm, less than about 100 nm, and less than about 50 nm. Such small effective average particle sizes can generally not be obtained using conventional mills.

As used herein, particle size is determined based on the weight average particle size as measured by conventional particle size measuring techniques well known to those skilled in the art. Such techniques include, for example, sedimentation field flow fractionation, photon correlation spectroscopy, light scattering, and disk centrifugation.

By "an effective average particle size of less than about 1 micron" it is meant that at least 50% of the candidate compound particles have an average particle size of less than about 1 micron when measured by the above techniques. Preferably, at least at least 60%, 70%, 80%, 90%, or 95% of the candidate compound particles are reduced to a particle size less than the effective average particle size, *i.e.*, less than about 1 micron, less than about 900 nm, less than about 800 nm.

#### **Concentration/Quantity of Candidate Compound; Dispersion Volume Required**

A small quantity of a candidate compound can be processed using the milling and microfluidization methods of the invention. For example, 100 mg of a candidate

compound (a 2% dispersion) can be used, and smaller amounts can also be used. Higher concentrations of candidate compound, at for example, 5% up to about 50%, can also be milled or microfluidized. 100 mg (2% dispersion) generally corresponds to 4-6 ml of total dispersion volume. The amount of candidate compound can be drug 5 dependent; for milling and microfluidization, the dispersion must be fluid and non-viscous.

In the methods of the invention, the candidate compound is present in a concentration selected from the group consisting of less than about 50%, less than about 40%, less than about 30%, less than about 25%, less than about 20%, less than about 10 15%, less than about 10%, less than about 5%, less than about 4%, less than about 3%, less than about 2%, less than about 1%, less than about 0.5%, less than about 0.1%, less than about 0.01%, and less than about 0.001%.

Alternatively, the candidate compound is present in an amount selected from the group consisting of from about 90% to about 0.001%, from about 90% to about 0.1%, 15 and from about 60% to about 5%, by weight, based on the total dry weight of the candidate compound and surface stabilizer.

The quantity of candidate compound required for the particle size reduction process is selected from the group consisting of less than about 100 mg, less than about 90 mg, less than about 80 mg, less than about 70 mg, less than about 60 mg, less than 20 about 50 mg, less than about 40 mg, less than about 30 mg, less than about 25 mg, less than about 20 mg, less than about 15 mg, less than about 10 mg, less than about 5 mg, less than about 4 mg, less than about 3 mg, less than about 2 mg less than about 1 mg, and less than about 0.5 mg.

In addition, the total dispersion volume required for the particle size reduction 25 process is selected from the group consisting of less than about 15 mL, less than about 10 mL, less than about 9 mL, less than about 8 mL, less than about 7 mL, less than about 6 mL, less than about 5 mL, less than about 4 mL, less than about 3 mL, and less than about 2 mL.

### **Small Scale Mill Structure**

A small-scale mill according to the present invention is designed to mill relatively small amounts of dispersion to a size ranging from microns to nanometers in a relatively short time, *i.e.*, one or more hours or less, using attrition milling media, such as polymeric media type, *e.g.*, cross linked polystyrene media, having a particle size of about 500 microns (0.5 mm) or less to about 50 microns, or mixtures of the sizes ranging between 500 and 50 microns.

A preferred small scale mill useful in the invention is described in WO 10 00/72973 for "Small Scale High Energy Mill," published on December 7, 2000.

Such a mill has few moving parts, has easy set up and clean up as it can be quickly dismantled, and it has a small footprint, which is critical in a laboratory setting. This is in contrast, for example, to Dymomill® mills which have many moving parts.

A preferred small scale mill is a table-top unit with a small foot-print, and several small scale mills can be used simultaneously to increase the screening of compounds.

In addition, a preferred small scale mill uses a water cooling system to allow for effective removal of excess heat generated during milling. In one embodiment, the cooling system can comprise a water jacket; in another embodiment, the mill chamber is double-walled to allow for circulation of coolant. In addition, three or more mills can be set up with a single cooling system. The presence of such a cooling systems allows for milling at higher speeds. Also, preferably the milling speed of the small scale mill can be varied. The combined effect of cooling and variability of milling speed makes the small scale mill an effective tool for temperature and/or milling energy sensitive compounds.

Yet another advantage of the preferred small scale mill is that rotors can be changed. Smooth shafts produce shear milling forces, while pegged shafts produce shear and impact forces. A pegged shaft is useful for a compound which is difficult to mill. Moreover, with the same milling head, different chamber sizes can be used, *i.e.*, chamber sizes of 10, 18, and 26 mls (in general, the dispersion size is about ½ of the

chamber size). This interchangeability of parts is a significant improvement over prior art milling technologies.

The rotor can be cylindrical, and can have tapered end surfaces. In one embodiment, the rotor is dimensioned so that its outer periphery is spaced no larger than 3 mm away from an inner surface of the vessel, particularly when the dispersion contains attrition media having a particle size of 500 microns or less. The spacing or the gap is preferably no larger than 1 mm, particularly when the dispersion contains attrition media having a particle size of 200 microns or less.

The vessel size can vary for milling small amounts of dispersion. Although the present invention is not limited to particular sizes, in a preferred embodiment the inner diameter of the vessel is between 5/8 inch to 4 inches. By way of example only, a milling chamber and a cylindrical rotor can have the dimensions specified in Tables 1 and 2.

15

<b>TABLE 1 (STRAIGHT ROTORS)</b>			
<b>CYLINDRICAL VESSEL Size</b>	<b>#1</b>	<b>#2</b>	<b>#3</b>
Volume Vessel (in <sup>3</sup> )	1.658	3.090	4.963
Volume Rotor (in <sup>3</sup> )	0.899	1.866	3.156
Volume Shaft (in <sup>3</sup> )	0.036	0.036	0.036
Working Volume (in <sup>3</sup> )	0.723	1.187	1.770
	11.855 ml	19.458 ml	29.012 ml
Typical Dispersion Volume @ 50% media charge	8.299 ml	13.621 ml	20.309 ml
Typical Dispersion Volume @ 90% media charge	5.453 ml	8.951 ml	13.346 ml

TABLE 2 (TAPERED ROTORS)			
VESSEL Size	#1	#2	#3
Volume Vessel (in <sup>3</sup> )	1.754	3.268	5.250
Volume Rotor Body (in <sup>3</sup> )	0.899	1.726	2.919
Volume Upper Cone (in <sup>3</sup> )	0.040	0.128	0.196
Volume Lower Cone (in <sup>3</sup> )	0.040	0.080	0.122
Volume Shaft (in <sup>3</sup> )	0.026	0.026	0.026
Volume Complete Rotor (in <sup>3</sup> )	0.979	1.934	3.237
Working Volume (in <sup>3</sup> )	0.749	1.308	1.986
	12.274 ml	21.429 ml	32.548 ml
Typical Dispersion Volume @ 50% media charge	8.592 ml	15.001 ml	22.784 ml
Typical Dispersion Volume @ 90% media charge	5.646 ml	9.858 ml	14.972 ml

\* \* \* \* \*

The following examples are given to illustrate the present invention. It should be understood, however, that the invention is not to be limited to the specific conditions or details described in these examples. Throughout the specification, any and all references to a publicly available documents are specifically incorporated into this patent application by reference.

#### 10 Example 1

The purpose of this example was to demonstrate the effectiveness of using high energy milling technology when formulating milligram quantities of poorly water soluble compounds for pre-clinical *in vivo* studies.

**Methods.** Small volume high-energy media mills capable of processing <100 mg of drug were tested for efficiency and performance using naproxen as the poorly water soluble drug candidate. A statistical design study was performed to test the robustness of the process and identify formulation parameters required to generate

nanoparticle suspensions having a mean particle size of less than 200 nm.

For processing, naproxen was milled in aqueous based stabilizer solutions at various concentrations for 15 min. to 60 min. at 10°C. The quality of the dispersion was evaluated using microscopy and laser light diffraction.

5 Naproxen has a molecular weight of 230.3 g, and a solubility in water of 16 µg/mL at pH 2 and 3.2 mg/mL at pH 7.5. The drug was milled at a low pH. The milling conditions were as follows: 3000-6000 rpm; 0.5% - 2% drug loading; 15 - 60 min. milling time; 60% - 90% media load; and 4 different mills were used (all were NanoMills™, manufactured by Elan Drug Delivery, Inc.).

10 A Greco Latin Square Design format was used and the experiment order was randomized. The table below shows the experiments performed:

<b>Table 3</b>					
<b>Experiment</b>	<b>Mill Speed (rpm)</b>	<b>Media Load (%)</b>	<b>Milling Time (min.)</b>	<b>Drug Conc. (%)</b>	<b>Mill #</b>
1	3000	70	30	1	2
2	3000	60	15	0.5	1
3	4000	70	15	2	3
4	3000	90	60	2	4
5	3000	80	45	1.5	3
6	4000	80	60	0.5	2
7	4000	60	30	1.5	4
8	6000	80	30	2	1
9	5000	80	15	1	4
10	4000	90	45	1	1
11	6000	90	15	1.5	2
12	5000	90	30	0.5	3
13	5000	70	60	1.5	1
14	5000	60	45	2	2
15	6000	60	60	1	3
16	6000	70	45	0.5	4

The important parameters for milling in a small scale mill are mill speed (rpm), 15 percent media load, drug concentration, the interaction between mill speed and media load, the interaction between milling time and drug concentration, and the interaction

between mill speed, percent media load, and drug concentration.

**Results.** The following preferred formulation and milling parameters were identified for obtaining a composition having a particle size of less than about 200 nm.

Mill Speed = 4300 rpm; minimum milling time = 15 min.; maximum milling time = 60 min.; minimum drug concentration = 0.5%; maximum drug concentration = 20%; and final yield was about 75%.

The study shows that a stable nanoparticle formulation of naproxen can be generated with < 50 mg of drug in 15 min. using a small-volume high energy mill. The nanoparticle suspensions were homogeneous as monitored by optical microscopy and exhibited a unimodal particle size distribution profile with a mean diameter of less than 200 nm. Approximately 90% of the drug was harvested after processing. Physical stability of the harvested formulations was acceptable after storage under refrigeration for at least two weeks.

15       **Conclusions.** Small-scale high energy wet-milling technology can be successfully utilized to generate stable formulations of poorly water soluble drugs in less than 15 min. with as little as 25 mg of drug. This approach provides an alternate method for effectively formulating poorly water soluble drugs that does not involve the use of solvents and is ideal for preclinical bioavailability and toxicology studies.

20

### **Example 2**

The purpose of this example was to demonstrate the reproducibility of the small scale milling process described in Example 1 using naproxen and several different new chemical entities.

25       Naproxen and five different poorly soluble new chemical entities having various chemistries, various mechanisms of action, and targeting different medical indications were milled as in Example 1. The results of the tests are shown below.

Table 4			
Drug	Amount Milled (mg)	Milling Time (min.)	Particle Size (nm)
Naproxen	50	15	159
Naproxen	200	60	147
Compound 1	200	60	93
Compound 2	200	60	166
Compound 3	200	60	162
Compound 4	200	60	188
Compound 5	200	60	168

The results demonstrate that the milling method is applicable to a wide variety of compounds, and is not limited by the chemical entity to be milled.

5

### Example 3

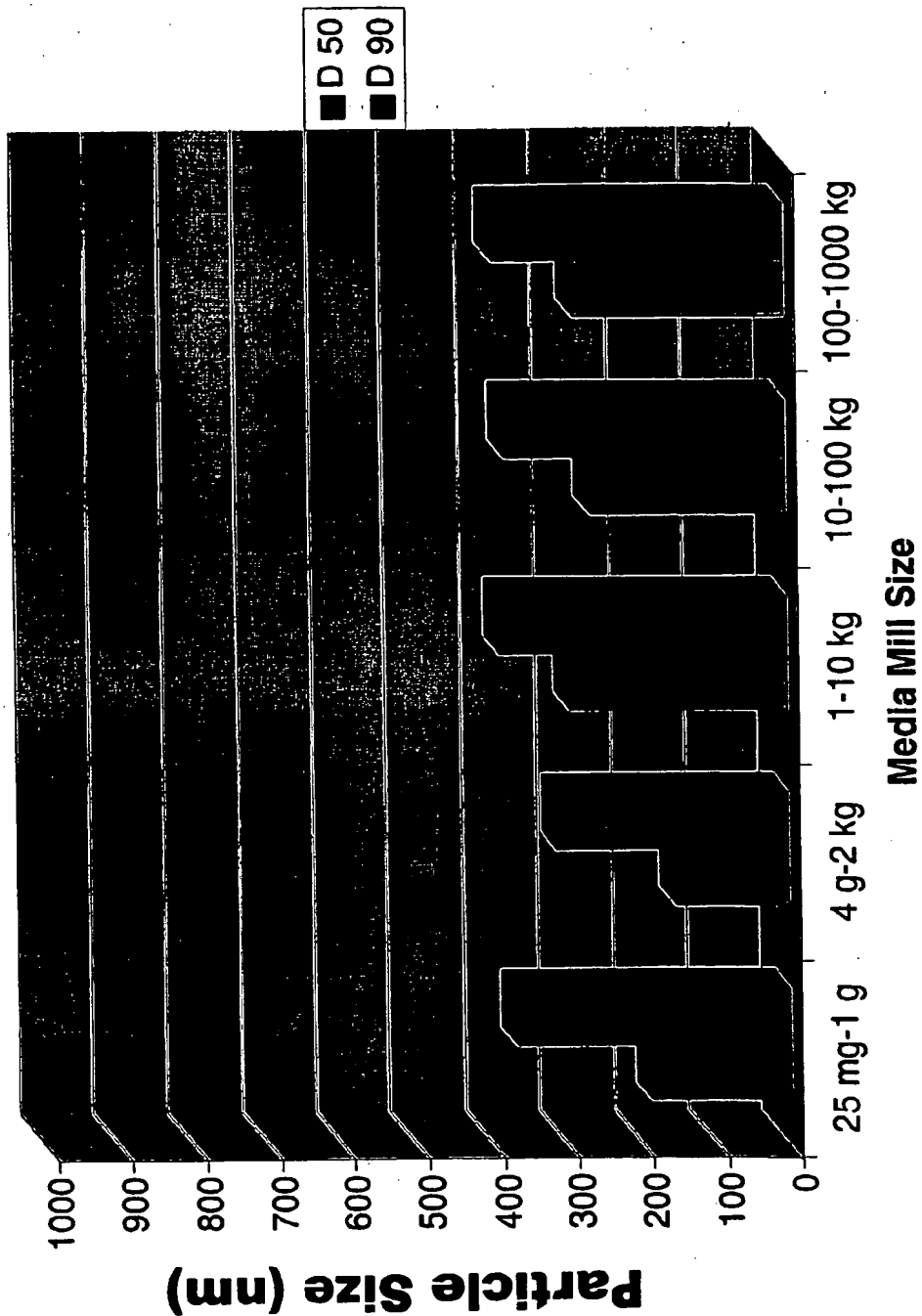
The purpose of this example was to demonstrate the effectiveness of scale-up of milling experiments conducted in a small scale mill to a large batch size milling process.

Naproxen was milled in five different media mill sizes: (1) 25 mg – 1 g; (2) 4 g 10 – 2 kg; (3) 1 – 10 kg; (4) 10 – 100 kg; and (5) 100 – 1000 kg. The results of the experiment are shown below. The value for D50 is the particle size below which 50% of the naproxen particles fall. Similarly, D90 is the particle size below which 90% of the naproxen particles fall.

15

Attorney Docket No. 29318/840

# Naproxen NanoCrystal Dispersion Scale-Up



The results given above demonstrate the consistency in particle size from milling in small quantities up to larger manufacturing scale quantities, particularly for D90 (*i.e.*, the particle size below which 90% of the particles of a composition fall). This is significant, as high throughput screening methods to identify suitable candidates for preclinical bioavailability and toxicology studies are significantly more useful if the screening methods used can be easily scaled up for manufacturing.

#### **Example 4**

The purpose of this example was to demonstrate the effectiveness of milling an extremely small quantity of active agent in a small scale mill.

About 15 mg of Compound X (0.5% drug) was combined with 0.25% Pluronic® F108 and 0.25% Na Deoxycholate for 60 minutes in a NanoMill™ (Elan Drug Delivery, Inc.). 6 mL of 0.8 mm YTZ grinding media (Yttria treated Zirconia; Tosoh Corporation) was used in the milling process.

The resultant formulation was well-dispersed and had an average particle size of about 300 nm, based on light microscopy analysis.

#### **Example 5**

The purpose of this example was to demonstrate successful small scale milling of very small quantities of drug.

Naproxen, polyvinylpyrrolidone (PVP) K29/32, and sodium lauryl sulfate (SLS) were combined in a ratio of 5:2:0.05, with naproxen present at 0.0625%. The mixture was milled in a NanoMill™ (Elan Drug Delivery, Inc.). Using a media load of 100% at the maximum rpm of 6000 maximized the energy input.

Further calculations were based on a bulk density of 0.61g/ml for PolyMill™ 500  $\mu$  media (Dow Chemical) and a void volume of 40%. Since the amounts were low and mixing difficult, PVP and SLS were prepared as 20% and 5% stock solutions, respectively. The calculations for milling in a 10 mL, 18 mL, and 26 mL milling chambers are summarized in the following chart:

<b>Table 5</b>			
	<b>10 mL chamber</b>	<b>18 mL chamber</b>	<b>26 mL chamber</b>
<b>RPM</b>	6000	6000	6000
<b>Media volume</b>	10 ml	18 ml	26 ml
<b>Media quantity; PolyMill™-500μ</b>	6.1 g	11.0 g	15.9 g
<b>Dispersion volume</b>	4.0 ml	7.2 ml	10.4 ml
<b>Naproxen</b>	25 mg	45 mg	65 mg
<b>PVP 29/32; 20%</b>	50 mg	90 mg	130 mg
<b>SLS; 5%</b>	5 mg	9 mg	13 mg
<b>Water For Injection</b>	3.92 g	7.06 g	10.27 g

10 ml chamber:

5        Analysis of the resultant particle size of the naproxen dispersion using a Horiba LA-910 Laser Scattering Particle Size Distribution Analyzer (Horiba Instruments, Irvine, CA) indicated that although a stable dispersion was formed, milling was not complete. A large aggregate peak of larger material was present in the particle sizing results. This peak decreased slightly over time, but was still  
10 present after 1 hr. The bimodal particle size peak had a mean of 1496 nm and a median of 354 nm. The median is therefore representative of the primary peak; the mean is higher due to the large aggregate of unmilled material still present in the sample.

15 18 ml chamber:

Complete milling of the naproxen dispersion was observed. Particle size analysis using a Horiba LA-910 Laser Scattering Particle Size Distribution Analyzer showed a particle size of essentially one single peak. The single peak mean particle size was 314 nm.

26 ml chamber:

Complete milling of the naproxen dispersion was observed. Particle size analysis using a Horiba LA-910 Laser Scattering Particle Size Distribution Analyzer showed a particle size of essentially one single peak, although the peak was much broader than the 18 mL sample. Narrower peaks correspond to a narrower particle size distribution. The single peak mean particle size was 630 nm.

Example 6

The purpose of this example was to mill a constant amount of active agent in 10 different size milling chambers to determine the effect on resultant active agent particle size. The effect of changing only the size of the milling chamber was to reduce the percentage of drug present.

25 mg samples of naproxen were milled in 10 mL, 18 mL, and 26 mL chambers of a NanoMill™. Naproxen, polyvinylpyrrolidone (PVP) K29/32, and 15 sodium lauryl sulfate (SLS) were combined in a ratio of 5:2:0.05. Analysis of resultant particle size was via photomicrography.

10 ml chamber:

A reasonable naproxen dispersion was formed after 15 min. Although a large population of larger and unmilled particles was clearly present, this was attributed to the chamber and rotor configuration and not the formulation. Later samples at 30 and 60 min. showed no improvement and were perhaps more heterogeneous.

18 ml chamber:

25 A reasonable naproxen dispersion was formed in 15 minutes, although the dispersion was more heterogeneous than that of the 10 ml chamber. The 30 min. sample was clearly over-milled and aggregated.

26 ml chamber:

Since the higher energy and/or greater time seemed to cause the sample to over-mill, this sample was viewed after 5 min. and showed a dispersion with excellent homogeneity, although larger particles were also present. After 15 min., the sample was clearly degenerating.

Examples 5 and 6 demonstrate that at extremely low levels of active agent, very little energy is required to achieve a "reasonable" dispersion that indicates whether or not a formulation screened was a viable candidate. In sum, in screening active agents to determine potential usefulness, use of a 10 mL chamber sampled at a 5 min. time point should be sufficient. Such a method has the advantage of avoiding over-milling of the sample if the dispersion is checked at later time points after continued milling.

Example 7

15 The purpose of this example is to demonstrate successful milling of an extremely small quantity of an active agent.

5 mg of naproxen, and PVP and SLS, were milled in a 10 mL chamber of a NanoMill™. Naproxen, PVP K29/32, and SLS were combined in a ratio of 5:2:0.05.

It was found that 5 mg of active agent, such as naproxen, in a 10 ml chamber  
20 was sufficient to form a dispersion that could be shown under photomicrography to be a well dispersed nano-suspension. Although larger particles were also present, this was attributed to the parameters used and the chamber and rotor configuration and not the formulation.

Thus, minute quantities, such as 5 mg, of active agent can be milled to determine  
25 the potential suitability of the composition for formulating in a nanoparticulate composition to increase bioavailability of the active agent.

**Example 8**

The purpose of this example is to demonstrate successful milling of an extremely small quantity of an active agent. A compound, Photogen (WIN 67722; 6-ethoxy-6-oxohexyl-3,5-bis(acetamido)-2,4,6-triiodobenzoate), was screened using the technique 5 of Example 7. Photogen is an iodinated imaging agent.

A mixture of 15% Photogen and 3% Pluronic® F-108 was adjusted to a ratio of 5:2 and decreased to 10 mg active agent. The composition was then milled in a 10 mL chamber of a NanoMill™. Although the resultant particle size was larger than naproxen, well-dispersed particles were seen in 5 min. and smaller particles were 10 formed in 15 and 30 min. The resultant particle size was predominantly sub-micron.

This example further demonstrates the usefulness of screening active agents in the drug discovery stage, when quantities of active agent may be limited, using a small scale mill or microfluidics, requiring small or minute quantities of active agent to produce nanoparticulate dispersions.

15

It will be apparent to those skilled in the art that various modifications and variations can be made in the methods of the present invention without departing from the spirit or scope of the invention. Thus, it is intended that the present invention cover the modifications and variations of this invention provided they come within the scope 20 of the appended claims and their equivalents.

## WE CLAIM:

1. A high throughput screening method comprising:
  - (a) reducing the particle size of one or more candidate compounds in a small scale mill in the presence of attrition milling media, wherein:
    - 5 (1) the one or more candidate compounds are milled in a liquid dispersion medium in which the candidate compounds are poorly soluble;
    - (2) the milled one or more candidate compounds have an effective average particle size of less than about 1 micron, and
    - 10 (3) at least one surface stabilizer is added to the liquid dispersion medium, either before or after particle size reduction, in an amount sufficient to maintain the effective average particle size of the one or more candidate compounds, following particle size reduction, at less than about 1 micron; and
  - 15 (b) screening the one or more nanoparticulate candidate compounds in a conventional high throughput screening assay to determine if the one or more compounds have a desired activity.
2. The method of claim 1, wherein the attrition milling media is polymeric.
- 20 3. The method of claim 1, wherein the attrition milling media has a particle size selected from the group consisting of about 500 microns or less, about 200 microns or less, about 50 microns or less, and mixtures thereof.
- 25 4. The method of claim 1, wherein the high throughput screening assay is an enzymatic or whole cell assay.
5. The method of claim 1, wherein the dispersion of nanoparticulate candidate compounds from step (a) is used directly in the high throughput screening

assay of step (b).

6. The method of claim 1, wherein the dispersion medium is selected from the group consisting of water, aqueous salt solutions, safflower oil, ethanol, t-butanol, 5 hexane, and glycol.

7. The method of claim 1, wherein the high throughput screening assay is manual or automatic.

10 8. The method of claim 1 in which a mixture of two or more candidate compounds is reduced in size in step (a).

9. The method of claim 1, wherein a mixture of two or more candidate compounds is screened in step (b).

15

10. The method of claim 1, wherein the candidate compound has a solubility in the liquid dispersion medium of less than about 10 mg/ml.

11. The method of claim 10, wherein the candidate compound has a 20 solubility in the liquid dispersion medium of less than about 1 mg/ml.

12. The method of claim 1, wherein the candidate compound is conjugated to a salt or other substance to render the candidate compound poorly soluble.

25 13. The method of claim 12, wherein the candidate compound is conjugated to a substance selected from the group consisting of hydrophobic molecules, molecules with amphipathic properties, lipid molecules, phospholipid molecules, fats, prenyl groups, and palmitoyl groups.

14. The method of claim 12 or 13, wherein such conjugation is accomplished by a method selected from the group consisting of direct conjugation to specific sites on the compound, conjugation to the N-terminal or C-terminal residue of the compound via intermediate spacer molecules, and conjugation through internal side chains on the 5 compound.

15. The method of claim 1, wherein the candidate compound is rendered poorly soluble by the addition of amino acid residues either during the chemical synthesis or the biological expression of the compound.

10

16. The method of claim 1, wherein the candidate compound is rendered poorly soluble by adjusting the pH of the dispersion medium.

17. The method of claim 1, wherein the candidate compound is selected from 15 the group consisting of a therapeutic agent, a cosmetic, a diagnostic agent, an agent useful in bioengineering, and an agricultural agent.

18. The method of claim 17, wherein the candidate compound is an agricultural agent selected from the group consisting of a pesticide, a fertilizer, an 20 insecticide, and a herbicide.

19. The method of claim 1, wherein the time between conducting step (a) and conducting step (b) extends for up to one year.

25 20. The method of claim 1, wherein the candidate compound is present in a concentration selected from the group consisting of less than about 50%, less than about 40%, less than about 30%, less than about 25%, less than about 20%, less than about 15%, less than about 10%, less than about 5%, less than about 4%, less than about 3%, less than about 2%, less than about 1%, less than about 0.5%, less than about 0.1%, less

than about 0.01%, and less than about 0.001%.

21. The method of claim 1, wherein the candidate compound is present in an amount selected from the group consisting of from about 90% to about 0.001%, from about 90% to about 0.1%, and from about 60% to about 5%, by weight, based on the total dry weight of the candidate compound and surface stabilizer.

22. The method of claim 1, wherein the quantity of candidate compound required for the particle size reduction process is selected from the group consisting of less than about 100 mg, less than about 90 mg, less than about 80 mg, less than about 70 mg, less than about 60 mg, less than about 50 mg, less than about 40 mg, less than about 30 mg, less than about 25 mg, less than about 20 mg, less than about 15 mg, less than about 10 mg, less than about 5 mg, less than about 4 mg, less than about 3 mg, less than about 2 mg, and less than about 1 mg.

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23. The method of claim 1, wherein the total dispersion volume required for the particle size reduction process is selected from the group consisting of less than about 15 mL, less than about 10 mL, less than about 9 mL, less than about 8 mL, less than about 7 mL, less than about 6 mL, less than about 5 mL, less than about 4 mL, less than about 3 mL, and less than about 2 mL.

24. The method of claim 1, wherein the time required for the particle size reduction process is selected from the group consisting of about one hour or less, about 45 minutes or less, about 40 minutes or less, about 35 minutes or less, about 30 minutes or less, about 25 minutes or less, about 20 minutes or less, about 15 minutes or less, about 10 minutes or less, and about 5 minutes or less.

25. The method of claim 1, wherein the at least one surface stabilizer is present in an amount selected from the group consisting of from about 0.01% to about

90%, from about 1% to about 90%, and from about 5% to about 90%, by weight, based on the total dry weight of the candidate compound and surface stabilizer.

26. The method of claim 1, wherein the at least one surface stabilizer is  
 5 selected from the group consisting of gelatin, casein, lecithin, dextran, gum acacia, cholesterol, tragacanth, stearic acid, benzalkonium chloride, calcium stearate, glycerol monostearate, cetostearyl alcohol, cetomacrogol emulsifying wax, sorbitan esters, polyoxyethylene alkyl ethers, polyoxyethylene castor oil derivatives, polyoxyethylene sorbitan fatty acid esters, polyethylene glycols, polyoxyethylene stearates, colloidal  
 10 silicon dioxide, phosphates, sodium dodecylsulfate, carboxymethylcellulose calcium, carboxymethylcellulose sodium, methylcellulose, hydroxyethylcellulose, hydroxypropylcellulose, hydroxypropylmethyl-cellulose phthalate, noncrystalline cellulose, magnesium aluminium silicate, triethanolamine, polyvinyl alcohol, polyvinylpyrrolidone, tyloxapol, poloxamers, poloxamines, Tetronic 1508<sup>®</sup>,  
 15 dialkylesters of sodium sulfosuccinic acid, sodium lauryl sulfates, alkyl aryl polyether sulfonates, a mixture of sucrose stearate and sucrose distearate, p-isononylphenoxypoly-(glycidol), Crodestas SL-40<sup>®</sup>, SA9OHCO which is  $C_{18}H_{37}CH_2C(O)N(CH_3)-CH_2(CHOH)_4(CH_2OH)_2$ , decanoyl-N-methylglucamide, n-decyl  $\beta$ -D-glucopyranoside, n-decyl  $\beta$ -D-maltopyranoside, n-dodecyl  $\beta$ -D-glucopyranoside, n-dodecyl  $\beta$ -D-  
 20 maltoside, heptanoyl-N-methylglucamide, n-heptyl- $\beta$ -D-glucopyranoside, n-heptyl  $\beta$ -D-thioglucoside, n-hexyl  $\beta$ -D-glucopyranoside, nonanoyl-N-methylglucamide, n-nonyl  $\beta$ -D-glucopyranoside, octanoyl-N-methylglucamide, n-octyl- $\beta$ -D-glucopyranoside, octyl  $\beta$ -D-thioglucopyranoside, PEG-phospholipid, PEG-cholesterol, PEG-cholesterol derivative, PEG-vitamin A, PEG-vitamin E, lysozyme, and random copolymers of vinyl  
 25 acetate and vinyl pyrrolidone.

27. The method of claim 1, wherein the candidate compound is reduced to an effective average particle size selected from the group consisting of less than about 900 nm, less than about 800 nm, less than about 700 nm, less than about 600 nm, less than

about 500 nm, less than about 400 nm, less than about 300 nm, less than about 250 nm, less than about 200 nm, less than about 150 nm, less than about 100 nm, and less than about 50 nm.

5           28.     The method of claim 25, wherein at least 60%, 70%, 80%, 90%, or 95% of the candidate compound particles are reduced to a particle size less than the effective average particle size.

          29.     A high throughput screening method comprising:

10           (a)     reducing the particle size of one or more candidate compounds using homogenization wherein:

                  (1)     the one or more candidate compounds are reduced in size in a liquid dispersion medium in which the candidate compounds are poorly soluble;

15                   (2)     the homogenized one or more candidate compounds have an effective average particle size of less than about 1 micron, and

                  (3)     at least one surface stabilizer is added to the liquid dispersion medium, either before or after particle size reduction, in an amount sufficient to maintain the effective average particle size  
20                   of the one or more candidate compounds, following particle size reduction, at less than about 1 micron; and

                  (b)     screening the one or more nanoparticulate candidate compounds in a conventional high throughput screening assay to determine if the one or more compounds have a desired activity.

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          30.     The method of claim 29, wherein the one or more candidate compounds are homogenized in the presence of attrition media.

          31.     The method of claim 30, wherein the attrition media is polymeric.

32. The method of claim 30, wherein the attrition media has a particle size selected from the group consisting of about 500 microns or less, about 200 microns or less, about 50 microns or less, and mixtures thereof.

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33. The method of claim 29, wherein the high throughput screening assay is an enzymatic or whole cell assay.

34. The method of claim 29, wherein the dispersion of nanoparticulate  
10 candidate compounds from step (a) is used directly in the high throughput screening assay of step (b).

35. The method of claim 29, wherein the dispersion medium is selected from the group consisting of water, aqueous salt solutions, safflower oil, ethanol, t-butanol,  
15 hexane, and glycol.

36. The method of claim 29, wherein the high throughput screening assay is manual or automatic.

20 37. The method of claim 29 in which a mixture of two or more candidate compounds is reduced in size in step (a).

38. The method of claim 29, wherein a mixture of two or more candidate compounds is screened in step (b).

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39. The method of claim 29, wherein the candidate compound has a solubility in the liquid dispersion medium of less than about 10 mg/ml.

40. The method of claim 39, wherein the candidate compound has a

solubility in the liquid dispersion medium of less than about 1 mg/ml.

41. The method of claim 29, wherein the candidate compound is conjugated to a salt or other substance to render the candidate compound poorly soluble.

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42. The method of claim 41, wherein the candidate compound is conjugated to a substance selected from the group consisting of hydrophobic molecules, molecules with amphipathic properties, lipid molecules, phospholipid molecules, fats, prenyl groups, and palmitoyl groups.

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43. The method of claim 41 or 42, wherein such conjugation is accomplished by a method selected from the group consisting of direct conjugation to specific sites on the compound, conjugation to the N-terminal or C-terminal residue of the compound via intermediate spacer molecules, and conjugation through internal side chains on the 15 compound.

44. The method of claim 29, wherein the candidate compound is rendered poorly soluble by the addition of amino acid residues either during the chemical synthesis or the biological expression of the compound.

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45. The method of claim 29, wherein the candidate compound is rendered poorly soluble by adjusting the pH of the dispersion medium.

46. The method of claim 29, wherein the candidate compound is selected 25 from the group consisting of a therapeutic agent, a cosmetic, a diagnostic agent, an agent useful in bioengineering, and an agricultural agent.

47. The method of claim 46, wherein the candidate compound is an agricultural agent selected from the group consisting of a pesticide, a fertilizer, an

insecticide, and a herbicide.

48. The method of claim 29, wherein the time between conducting step (a) and conducting step (b) extends for up to one year.

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49. The method of claim 29, wherein the candidate compound is present in a concentration selected from the group consisting of less than about 50%, less than about 40%, less than about 30%, less than about 25%, less than about 20%, less than about 15%, less than about 10%, less than about 5%, less than about 4%, less than about 3%, less than about 2%, less than about 1%, less than about 0.5%, less than about 0.1%, less than about 0.01%, and less than about 0.001%.

50. The method of claim 29, wherein the candidate compound is present in an amount selected from the group consisting of from about 90% to about 0.001%, from about 90% to about 0.1%, and from about 60% to about 5%, by weight, based on the total dry weight of the candidate compound and surface stabilizer.

51. The method of claim 29, wherein the quantity of candidate compound required for the particle size reduction process is selected from the group consisting of less than about 100 mg, less than about 90 mg, less than about 80 mg, less than about 70 mg, less than about 60 mg, less than about 50 mg, less than about 40 mg, less than about 30 mg, less than about 25 mg, less than about 20 mg, less than about 15 mg, less than about 10 mg, less than about 5 mg, less than about 4 mg, less than about 3 mg, less than about 2 mg, and less than about 1 mg.

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52. The method of claim 29, wherein the total dispersion volume required for the particle size reduction process is selected from the group consisting of less than about 15 mL, less than about 10 mL, less than about 9 mL, less than about 8 mL, less than about 7 mL, less than about 6 mL, less than about 5 mL, less than about 4 mL, less

than about 3 mL, and less than about 2 mL.

53. The method of claim 29, wherein the time required for the particle size reduction process is selected from the group consisting of about one hour or less, about 5 45 minutes or less, about 40 minutes or less, about 35 minutes or less, about 30 minutes or less, about 25 minutes or less, about 20 minutes or less, about 15 minutes or less, about 10 minutes or less, and about 5 minutes or less.

54. The method of claim 29, wherein the at least one surface stabilizer is 10 present in an amount selected from the group consisting of from about 0.01% to about 90%, from about 1% to about 90%, and from about 5% to about 90%, by weight, based on the total dry weight of the candidate compound and surface stabilizer.

55. The method of claim 29, wherein the at least one surface stabilizer is 15 selected from the group consisting of gelatin, casein, lecithin, dextran, gum acacia, cholesterol, tragacanth, stearic acid, benzalkonium chloride, calcium stearate, glycerol monostearate, cetostearyl alcohol, cetomacrogol emulsifying wax, sorbitan esters, polyoxyethylene alkyl ethers, polyoxyethylene castor oil derivatives, polyoxyethylene sorbitan fatty acid esters, polyethylene glycols, polyoxyethylene stearates, colloidal 20 silicon dioxide, phosphates, sodium dodecylsulfate, carboxymethylcellulose calcium, carboxymethylcellulose sodium, methylcellulose, hydroxyethylcellulose, hydroxypropylcellulose, hydroxypropylmethyl-cellulose phthalate, noncrystalline cellulose, magnesium aluminium silicate, triethanolamine, polyvinyl alcohol, polyvinylpyrrolidone, tyloxapol, poloxamers, poloxamines, Tetronic 1508®, 25 dialkylesters of sodium sulfosuccinic acid, sodium lauryl sulfates, alkyl aryl polyether sulfonates, a mixture of sucrose stearate and sucrose distearate, p-isononylphenoxypoly-(glycidol), Crodestas SL-40®, SA9OHCO which is  $C_{18}H_{37}CH_2C(O)N(CH_3)-CH_2(CHOH)_4(CH_2OH)_2$ , decanoyl-N-methylglucamide, n-decyl  $\beta$ -D-glucopyranoside, n-decyl  $\beta$ -D-maltopyranoside, n-dodecyl  $\beta$ -D-glucopyranoside, n-dodecyl  $\beta$ -D-

maltoside, heptanoyl-N-methylglucamide, n-heptyl- $\beta$ -D-glucopyranoside, n-heptyl  $\beta$ -D-thioglucoside, n-hexyl  $\beta$ -D-glucopyranoside, nonanoyl-N-methylglucamide, n-nonyl  $\beta$ -D-glucopyranoside, octanoyl-N-methylglucamide, n-octyl- $\beta$ -D-glucopyranoside, octyl  $\beta$ -D-thioglucopyranoside, PEG-phospholipid, PEG-cholesterol, PEG-cholesterol derivative, PEG-vitamin A, PEG-vitamin E, lysozyme, and random copolymers of vinyl acetate and vinyl pyrrolidone.

56. The method of claim 29, wherein the candidate compound is reduced to an effective average particle size selected from the group consisting of less than about 900 nm, less than about 800 nm, less than about 700 nm, less than about 600 nm, less than about 500 nm, less than about 400 nm, less than about 300 nm, less than about 250 nm, less than about 200 nm, less than about 150 nm, less than about 100 nm, and less than about 50 nm.

57. The method of claim 56, wherein at least 60%, 70%, 80%, 90%, or 95% of the candidate compound particles are reduced to a particle size less than the effective average particle size.

58. A high throughput screening method comprising:

(a) screening one or more candidate compounds in a conventional high throughput screening assay to determine if the one or more compounds have a desired activity; and

(b) reducing the particle size of the one or more candidate compounds in a small scale mill in the presence of attrition milling media, wherein:

- (1) the one or more candidate compounds are milled in a liquid dispersion medium in which the candidate compounds are poorly soluble;
- (2) the milled one or more compounds have an effective average particle size of less than about 1 micron, and

- 5
- (3) at least one surface stabilizer is added to the liquid dispersion medium, either before or after particle size reduction, in an amount sufficient to maintain the effective average particle size of the one or more candidate compounds, following particle size reduction, at less than about 1 micron, and
  - (4) determining if the one or more compounds have acceptable solubility and/or dispersibility.

10 59. The method of claim 58, wherein the attrition milling media is polymeric.

15 60. The method of claim 58, wherein the attrition milling media has a particle size selected from the group consisting of about 500 microns or less, about 200 microns or less, about 50 microns or less, and mixtures thereof.

61. The method of claim 58, wherein the high throughput screening assay is an enzymatic or whole cell assay.

20 62. The method of claim 58, wherein the dispersion medium is selected from the group consisting of water, aqueous salt solutions, safflower oil, ethanol, t-butanol, hexane, and glycol.

63. The method of claim 58, wherein the high throughput screening assay is manual or automatic.

25 64. The method of claim 58 in which a mixture of two or more candidate compounds is reduced in size in step (b).

65. The method of claim 58, wherein a mixture of two or more candidate

compounds is screened in step (a).

66. The method of claim 58, wherein the candidate compound has a solubility in the liquid dispersion medium of less than about 10 mg/ml.

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67. The method of claim 66, wherein the candidate compound has a solubility in the liquid dispersion medium of less than about 1 mg/ml.

68. The method of claim 58, wherein the candidate compound is conjugated to a salt or other substance to render the candidate compound poorly soluble.

69. The method of claim 68, wherein the candidate compound is conjugated to a substance selected from the group consisting of hydrophobic molecules, molecules with amphipathic properties, lipid molecules, phospholipid molecules, fats, prenyl groups, and palmitoyl groups.

70. The method of claim 68 or 69, wherein such conjugation is accomplished by a method selected from the group consisting of direct conjugation to specific sites on the compound, conjugation to the N-terminal or C-terminal residue of the compound via intermediate spacer molecules, and conjugation through internal side chains on the compound.

71. The method of claim 58, wherein the candidate compound is rendered poorly soluble by the addition of amino acid residues either during the chemical synthesis or the biological expression of the compound.

72. The method of claim 58, wherein the candidate compound is rendered poorly soluble by adjusting the pH of the dispersion medium.

73. The method of claim 58, wherein the candidate compound is selected from the group consisting of a therapeutic agent, a cosmetic, a diagnostic agent, an agent useful in bioengineering, and an agricultural agent.

5 74. The method of claim 73, wherein the candidate compound is an agricultural agent selected from the group consisting of a pesticide, a fertilizer, an insecticide, and a herbicide.

75. The method of claim 58, wherein the time between conducting step (a) 10 and conducting step (b) extends for up to one year.

76. The method of claim 58, wherein the candidate compound is present in a concentration selected from the group consisting of less than about 50%, less than about 40%, less than about 30%, less than about 25%, less than about 20%, less than about 15 15%, less than about 10%, less than about 5%, less than about 4%, less than about 3%, less than about 2%, less than about 1%, less than about 0.5%, less than about 0.1%, less than about 0.01%, and less than about 0.001%.

77. The method of claim 58, wherein the candidate compound is present in 20 an amount selected from the group consisting of from about 90% to about 0.001%, from about 90% to about 0.1%, and from about 60% to about 5%, by weight, based on the total dry weight of the candidate compound and surface stabilizer.

78. The method of claim 58, wherein the quantity of candidate compound 25 required for the particle size reduction process is selected from the group consisting of less than about 100 mg, less than about 90 mg, less than about 80 mg, less than about 70 mg, less than about 60 mg, less than about 50 mg, less than about 40 mg, less than about 30 mg, less than about 25 mg, less than about 20 mg, less than about 15 mg, less than about 10 mg, less than about 5 mg, less than about 4 mg, less than about 3 mg, less than

about 2 mg, and less than about 1 mg.

79. The method of claim 58, wherein the total dispersion volume required for the particle size reduction process is selected from the group consisting of less than about 15 mL, less than about 10 mL, less than about 9 mL, less than about 8 mL, less than about 7 mL, less than about 6 mL, less than about 5 mL, less than about 4 mL, less than about 3 mL, and less than about 2 mL.

80. The method of claim 58, wherein the time required for the particle size reduction process is selected from the group consisting of about one hour or less, about 45 minutes or less, about 40 minutes or less, about 35 minutes or less, about 30 minutes or less, about 25 minutes or less, about 20 minutes or less, about 15 minutes or less, about 10 minutes or less, and about 5 minutes or less.

81. The method of claim 58, wherein the at least one surface stabilizer is present in an amount selected from the group consisting of from about 0.01% to about 90%, from about 1% to about 90%, and from about 5% to about 90%, by weight, based on the total dry weight of the candidate compound and surface stabilizer.

82. The method of claim 58, wherein the at least one surface stabilizer is selected from the group consisting of gelatin, casein, lecithin, dextran, gum acacia, cholesterol, tragacanth, stearic acid, benzalkonium chloride, calcium stearate, glycerol monostearate, cetostearyl alcohol, cetomacrogol emulsifying wax, sorbitan esters, polyoxyethylene alkyl ethers, polyoxyethylene castor oil derivatives, polyoxyethylene sorbitan fatty acid esters, polyethylene glycols, polyoxyethylene stearates, colloidal silicon dioxide, phosphates, sodium dodecylsulfate, carboxymethylcellulose calcium, carboxymethylcellulose sodium, methylcellulose, hydroxyethylcellulose, hydroxypropylcellulose, hydroxypropylmethyl-cellulose phthalate, noncrystalline cellulose, magnesium aluminium silicate, triethanolamine, polyvinyl alcohol,

polyvinylpyrrolidone, tyloxapol, poloxamers, poloxamines, Tetronic 1508<sup>®</sup>, dialkylesters of sodium sulfosuccinic acid, sodium lauryl sulfates, alkyl aryl polyether sulfonates, a mixture of sucrose stearate and sucrose distearate, p-isononylphenoxypoly-(glycidol), Crodestas SL-40<sup>®</sup>, SA9OHCO which is  $C_{18}H_{37}CH_2C(O)N(CH_3)-$   
 5  $CH_2(CHOH)_4(CH_2OH)_2$ , decanoyl-N-methylglucamide, n-decyl  $\beta$ -D-glucopyranoside, n-decyl  $\beta$ -D-maltopyranoside, n-dodecyl  $\beta$ -D-glucopyranoside, n-dodecyl  $\beta$ -D-maltoside, heptanoyl-N-methylglucamide, n-heptyl- $\beta$ -D-glucopyranoside, n-heptyl  $\beta$ -D-thioglucoside, n-hexyl  $\beta$ -D-glucopyranoside, nonanoyl-N-methylglucamide, n-nonyl  $\beta$ -D-glucopyranoside, octanoyl-N-methylglucamide, n-octyl- $\beta$ -D-glucopyranoside, octyl  
 10  $\beta$ -D-thioglucopyranoside, PEG-phospholipid, PEG-cholesterol, PEG-cholesterol derivative, PEG-vitamin A, PEG-vitamin E, lysozyme, and random copolymers of vinyl acetate and vinyl pyrrolidone.

83. The method of claim 58, wherein the candidate compound is reduced to  
 15 an effective average particle size selected from the group consisting of less than about 900 nm, less than about 800 nm, less than about 700 nm, less than about 600 nm, less than about 500 nm, less than about 400 nm, less than about 300 nm, less than about 250 nm, less than about 200 nm, less than about 150 nm, less than about 100 nm, and less than about 50 nm.

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84. The method of claim 83, wherein at least 60%, 70%, 80%, 90%, or 95% of the candidate compound particles are reduced to a particle size less than the effective average particle size.

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85. A high throughput screening method comprising:

- (a) screening one or more candidate compounds in a conventional high throughput screening assay to determine if the one or more compounds have a desired activity; and
- (b) reducing the particle size of the one or more candidate compounds in a

small scale mill in the presence of attrition milling media, wherein:

- (1) the one or more candidate compounds are milled in a liquid dispersion medium in which the candidate compounds are poorly soluble;
- 5 (2) the milled one or more compounds have an effective average particle size of less than about 1 micron, and
- (3) at least one surface stabilizer is added to the liquid dispersion medium, either before or after particle size reduction, in an amount sufficient to maintain the effective average particle size
- 10 of the one or more candidate compounds, following particle size reduction, at less than about 1 micron, and
- (4) determining if the one or more compounds have acceptable solubility and/or dispersibility.

15 86. The method of claim 85, wherein the one or more candidate compounds are homogenized in the presence of attrition media.

87. The method of claim 86, wherein the attrition media is polymeric.

20 88. The method of claim 86, wherein the attrition media has a particle size selected from the group consisting of about 500 microns or less, about 200 microns or less, about 50 microns or less, and mixtures thereof.

89. The method of claim 85, wherein the high throughput screening assay is  
25 an enzymatic or whole cell assay.

90. The method of claim 85, wherein the dispersion of nanoparticulate candidate compounds from step (a) is used directly in the high throughput screening assay of step (b).

91. The method of claim 85, wherein the dispersion medium is selected from the group consisting of water, aqueous salt solutions, safflower oil, ethanol, t-butanol, hexane, and glycol.

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92. The method of claim 85, wherein the high throughput screening assay is manual or automatic.

93. The method of claim 85 in which a mixture of two or more candidate  
10 compounds is reduced in size in step (b).

94. The method of claim 85, wherein a mixture of two or more candidate compounds is screened in step (a).

15 95. The method of claim 85, wherein the candidate compound has a solubility in the liquid dispersion medium of less than about 10 mg/ml.

96. The method of claim 95, wherein the candidate compound has a solubility in the liquid dispersion medium of less than about 1 mg/ml.

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97. The method of claim 85, wherein the candidate compound is conjugated to a salt or other substance to render the candidate compound poorly soluble.

98. The method of claim 97, wherein the candidate compound is conjugated  
25 to a substance selected from the group consisting of hydrophobic molecules, molecules with amphipathic properties, lipid molecules, phospholipid molecules, fats, prenyl groups, and palmitoyl groups.

99. The method of claim 97 or 98, wherein such conjugation is accomplished

by a method selected from the group consisting of direct conjugation to specific sites on the compound, conjugation to the N-terminal or C-terminal residue of the compound via intermediate spacer molecules, and conjugation through internal side chains on the compound.

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100. The method of claim 85, wherein the candidate compound is rendered poorly soluble by the addition of amino acid residues either during the chemical synthesis or the biological expression of the compound.

101. The method of claim 85, wherein the candidate compound is rendered poorly soluble by adjusting the pH of the dispersion medium.

102. The method of claim 85, wherein the candidate compound is selected from the group consisting of a therapeutic agent, a cosmetic, a diagnostic agent, an agent useful in bioengineering, and an agricultural agent.

103. The method of claim 102, wherein the candidate compound is an agricultural agent selected from the group consisting of a pesticide, a fertilizer, an insecticide, and a herbicide.

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104. The method of claim 85, wherein the time between conducting step (a) and conducting step (b) extends for up to one year.

105. The method of claim 85, wherein the candidate compound is present in a concentration selected from the group consisting of less than about 50%, less than about 40%, less than about 30%, less than about 25%, less than about 20%, less than about 15%, less than about 10%, less than about 5%, less than about 4%, less than about 3%, less than about 2%, less than about 1%, less than about 0.5%, less than about 0.1%, less than about 0.01%, and less than about 0.001%.

106. The method of claim 85, wherein the candidate compound is present in an amount selected from the group consisting of from about 90% to about 0.001%, from about 90% to about 0.1%, and from about 60% to about 5%, by weight, based on the 5 total dry weight of the candidate compound and surface stabilizer.

107. The method of claim 85, wherein the quantity of candidate compound required for the particle size reduction process is selected from the group consisting of less than about 100 mg, less than about 90 mg, less than about 80 mg, less than about 70 mg, less than about 60 mg, less than about 50 mg, less than about 40 mg, less than about 30 mg, less than about 25 mg, less than about 20 mg, less than about 15 mg, less than about 10 mg, less than about 5 mg, less than about 4 mg, less than about 3 mg, less than about 2 mg, and less than about 1 mg.

15 108. The method of claim 85, wherein the total dispersion volume required for the particle size reduction process is selected from the group consisting of less than about 15 mL, less than about 10 mL, less than about 9 mL, less than about 8 mL, less than about 7 mL, less than about 6 mL, less than about 5 mL, less than about 4 mL, less than about 3 mL, and less than about 2 mL.

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109. The method of claim 85, wherein the time required for the particle size reduction process is selected from the group consisting of about one hour or less, about 45 minutes or less, about 40 minutes or less, about 35 minutes or less, about 30 minutes or less, about 25 minutes or less, about 20 minutes or less, about 15 minutes or less, 25 about 10 minutes or less, and about 5 minutes or less.

110. The method of claim 85, wherein the at least one surface stabilizer is present in an amount selected from the group consisting of from about 0.01% to about 90%, from about 1% to about 90%, and from about 5% to about 90%, by weight, based

on the total dry weight of the candidate compound and surface stabilizer.

111. The method of claim 85, wherein the at least one surface stabilizer is selected from the group consisting of gelatin, casein, lecithin, dextran, gum acacia, 5 cholesterol, tragacanth, stearic acid, benzalkonium chloride, calcium stearate, glycerol monostearate, cetostearyl alcohol, cetomacrogol emulsifying wax, sorbitan esters, polyoxyethylene alkyl ethers, polyoxyethylene castor oil derivatives, polyoxyethylene sorbitan fatty acid esters, polyethylene glycols, polyoxyethylene stearates, colloidal silicon dioxide, phosphates, sodium dodecylsulfate, carboxymethylcellulose calcium, 10 carboxymethylcellulose sodium, methylcellulose, hydroxyethylcellulose, hydroxypropylcellulose, hydroxypropylmethyl-cellulose phthalate, noncrystalline cellulose, magnesium aluminium silicate, triethanolamine, polyvinyl alcohol, polyvinylpyrrolidone, tyloxapol, poloxamers, poloxamines, Tetronic 1508<sup>®</sup>, dialkylesters of sodium sulfosuccinic acid, sodium lauryl sulfates, alkyl aryl polyether 15 sulfonates, a mixture of sucrose stearate and sucrose distearate, p-isononylphenoxypoly-(glycidol), Crodestas SL-40<sup>®</sup>, SA9OHCO which is  $C_{18}H_{37}CH_2C(O)N(CH_3)-CH_2(CHOH)_4(CH_2OH)_2$ , decanoyl-N-methylglucamide, n-decyl  $\beta$ -D-glucopyranoside, n-decyl  $\beta$ -D-maltopyranoside, n-dodecyl  $\beta$ -D-glucopyranoside, n-dodecyl  $\beta$ -D-maltoside, heptanoyl-N-methylglucamide, n-heptyl- $\beta$ -D-glucopyranoside, n-heptyl  $\beta$ -D- 20 thioglucoside, n-hexyl  $\beta$ -D-glucopyranoside, nonanoyl-N-methylglucamide, n-nonyl  $\beta$ -D-glucopyranoside, octanoyl-N-methylglucamide, n-octyl- $\beta$ -D-glucopyranoside, octyl  $\beta$ -D-thioglucopyranoside, PEG-phospholipid, PEG-cholesterol, PEG-cholesterol derivative, PEG-vitamin A, PEG-vitamin E, lysozyme, and random copolymers of vinyl acetate and vinyl pyrrolidone.

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112. The method of claim 85, wherein the candidate compound is reduced to an effective average particle size selected from the group consisting of less than about 900 nm, less than about 800 nm, less than about 700 nm, less than about 600 nm, less than about 500 nm, less than about 400 nm, less than about 300 nm, less than about 250

nm, less than about 200 nm, less than about 150 nm, less than about 100 nm, and less than about 50 nm.

113. The method of claim 112, wherein at least 60%, 70%, 80%, 90%, or 95% 5 of the candidate compound particles are reduced to a particle size less than the effective average particle size.

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- (71) Applicant and  
(72) Inventor: **LINDNER, Marie** [US/US]; 631 Hollow Road,  
Radnor, PA 19087 (US).
- (72) Inventors; and  
(75) Inventors/Applicants (for US only): **MERISKO-LIV-ERSIDGE, Elaine** [US/US]; 258 Colwyn Terr., West Chester, PA 19380 (US). **GOTTARDY, Greta** [US/US]; 204 Marlbrook Lane, Lansdale, PA 19446 (US).
- (74) Agents: **SIMKIN, Michele, M.** et al.; Foley & Lardner, 3000 K Street, N.W., Suite 500, Washington, DC 20007-5143 (US).
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- Published:  
— with international search report
- (88) Date of publication of the international search report:  
18 December 2003
- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: **METHOD FOR HIGH THROUGH PUT SCREENING USING A SMALL SCALE MILL OR MICROFLUIDICS**

(57) Abstract: The present invention is directed to a high throughput screening (HTS) method, comprising reducing the particle size of a poorly soluble candidate compound to about 1 micron or less using a small scale mill or microfluidics. The product produced from this process is a dispersion of a nanoparticulate candidate compound having one or more surface stabilizers adsorbed onto the surface of the compound. The reduction in particle size results in an increase in the solubility and/or dispersibility of the compound, thus increasing the effectiveness of HTS conducted in conjunction with the particle size reduction process. The particle size reduction process can be conducted before HTS to make screening compounds soluble and/or dispersible, or after HTS to validate an insoluble or poorly compound determined to be active after screening.

WO 03/000228 A3

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 02/16589

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 A61K9/14

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, MEDLINE

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5 862 999 A (CZEKAI DAVID A ET AL) 26 January 1999 (1999-01-26) the whole document	1-28
A	US 5 718 388 A (CZEKAI DAVID A ET AL) 17 February 1998 (1998-02-17) the whole document	1-28
A	US 5 510 118 A (SWANSON JON R ET AL) 23 April 1996 (1996-04-23) the whole document	1-28

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

### \* Special categories of cited documents :

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
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\*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

\*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

\*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

\*G\* document member of the same patent family

Date of the actual completion of the international search

15 May 2003

Date of mailing of the international search report

26/05/2003

Name and mailing address of the ISA  
European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax: (+31-70) 340-3016

Authorized officer

Uhl, M

## FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 29-113

In view of the large number and also the wording of the claims presently on file, which render it difficult, if not impossible, to determine the matter for which protection is sought, the present application fails to comply with the clarity and conciseness requirements of Article 6 PCT (see also Rule 6.1(a) PCT) to such an extent that a meaningful search is impossible. Consequently, the search has been carried out for those parts of the application which do appear to be clear (and concise), namely concerning subject matter of claims 1-28.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US 02/16589

### Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☒ Claims Nos.: 29-113  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:  
see FURTHER INFORMATION sheet PCT/ISA/210
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

### Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this International application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 02/16589

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
US 5862999	A	26-01-1999	AT 196601 T	15-10-2000
			AU 2550195 A	18-12-1995
			CA 2190966 A1	30-11-1995
			DE 69518981 D1	02-11-2000
			DE 69518981 T2	01-03-2001
			DK 760653 T3	18-12-2000
			EP 0760653 A1	12-03-1997
			ES 2152401 T3	01-02-2001
			GR 3035040 T3	30-03-2001
			JP 10501709 T	17-02-1998
			PT 760653 T	30-03-2001
			TW 384224 B	11-03-2000
			WO 9531974 A1	30-11-1995
US 5718388	A	17-02-1998	AT 208191 T	15-11-2001
			AU 2476195 A	18-12-1995
			CA 2190134 A1	30-11-1995
			DE 69523781 D1	13-12-2001
			DE 69523781 T2	13-06-2002
			DK 804161 T3	04-03-2002
			EP 0804161 A1	05-11-1997
			ES 2165913 T3	01-04-2002
			IL 113851 A	22-12-1999
			JP 10500593 T	20-01-1998
			PT 804161 T	29-04-2002
			TW 460297 B	21-10-2001
			WO 9531973 A1	30-11-1995
US 5510118	A	23-04-1996	AU 4867396 A	04-09-1996
			WO 9625152 A1	22-08-1996

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